

# Major Loss of the 28-kD Protein of Gap Junction in Proliferating Hepatocytes

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**Abstract.** There is a reduction in the 28-kD gap junction protein detectable by immunofluorescence in livers of partially hepatectomized rats and in cultured hepatocytes stimulated to proliferate. By the coordinate use of antibodies directed to the hepatic junction protein (HJP28) and the use of a monoclonal antibody that recognizes bromodeoxyuridine (BrdU) incorporated into DNA, we have been able to study the relationship between detectable gap junction protein and cell divi-

sion. Hepatocytes that label with BrdU in the regenerating liver and in cell culture show a significant reduction of HJP28. Cells that do not synthesize DNA, on the other hand, show normal levels and distribution of immunoreactive gap junction protein. We postulate that the quantitative changes in gap junction expression might play an important role in the control of proliferation in the liver.

**G**AP junctions represent a class of intercellular contacts which are commonly assumed to be the site of exchange of inorganic ions and small molecules (3, 4, 14, 35). It has been suggested that they may be involved in regulating various cellular activities such as growth control (7, 27), and the modulation of developmental processes during embryogenesis (19, 38, 39, 44). There is a close correlation between the ultrastructural demonstration of gap junctions and the presence of cell-cell coupling measured electrophysiologically or by injection of fluorescent dyes (2, 14, 36). Several laboratories have recently reported the characterization of antibodies directed against the 28-kD protein that is a major component of isolated rat liver gap junctions (hepatic junction protein, HJP28)<sup>1</sup> (9, 20, 23, 24, 32, 42). Intracellular injection of specific antibodies raised against rodent HJP28 inhibits electrical or dye-coupling between several types of vertebrate cells (21, 28). Injection of antibodies raised against this protein into blastomeres of *Xenopus* appears to cause developmental defects in the larva (43). These antibodies thus detect a functionally competent form of HJP28.

The regenerating liver is an interesting experimental model where the possible correlation between gap junctions and the control of growth can be examined (12, 29, 41, 45, 46). Partial hepatectomy causes a burst of cell division that, by morphometric studies of freeze-cleaved material, has been shown to be temporally correlated with a cycle of disappearance and reappearance of gap junctions (45, 46). A significant reduction of the HJP28 has also been seen after partial hepatectomy by biochemical and immunochemical criteria (12, 41).

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; HJP, hepatic junction protein.

Not all cells participate in this wave of cell proliferation (34) nor is there a complete loss of junctional communication (29). Until now it has not been possible to establish a definite link between cell division and the loss of gap junction. The experiments described here use a double-immunolabeling approach to the study of regenerating liver cells and of cultured primary hepatocytes from embryonic mice (8). We label HJP28 with a monospecific antibody (42), and simultaneously detect cells that have been in S phase with an antibody directed to bromodeoxyuridine (BrdU) (11, 17), enabling us to determine the presence or absence of gap junctions on individual cells for which the position in the cell cycle is known.

## Materials and Methods

### Partial Hepatectomy

3–4-wk-old Sprague-Dawley rats were partially hepatectomized under ether anesthesia according to the method described by Higgins and Anderson (22). The median and left lateral lobes were removed and the others minimally disturbed and left in situ. The incisions were sutured and the animals allowed to recover with free access to food and water. Sham-operated animals were treated in the same fashion except that the liver was only manipulated with no portion of it removed. One control and two experimental animals were killed by cervical dislocation at 19-, 21-, 24-, 26-, 28-, 30-, 32-, and 34-h intervals after the hepatectomy. After flushing the liver vasculature with Ringer-0.1% heparin solution, the right lateral lobe was removed immediately, sliced, and the slices were frozen in liquid propane maintained at its freezing point. To label cells that were going through S phase before each experimental time point, a BrdU tablet (50 mg; Boehringer Mannheim Diagnostics, Inc., Houston, TX) was implanted subcutaneously, 7 h before killing. This period corresponds to the duration of the S phase in regenerating hepatocytes (34). Implantation of BrdU tablets ensured continued high levels of labeling without necessitating multiple in-

jections, or tail vein infusion (1), manipulations that can interfere with the replication kinetics of the liver cells (6).

### Culture of Embryonic Mouse Hepatocytes

For isolation and culture of embryonic mouse hepatocytes, livers from ~50 embryos (18 d old) of NMRI mice were cut into small pieces in 10 ml of "attachment" medium (see below). The pieces were slowly stirred in 20 ml of PBS containing collagenase (type 1; 0.63 mg per ml PBS; Sigma Chemical Co., St. Louis, MO) for 10 min at 37°C. After settling at room temperature for 2 min the supernatant was pipetted into 20 ml of ice-cold attachment medium. The collagenase treatment was repeated three times and the pooled supernatants were centrifuged for 15 s (at 950 g) in order to pellet only the relatively large hepatocytes (yield:  $\sim 5 \times 10^7$  cells). About  $5 \times 10^6$  cells were plated on 60-mm dish that contained three or four glass coverslips (16  $\times$  16 mm). After 3 h at 37°C the attachment medium was replaced by chemically defined, arginine-free MX83 medium, containing 1  $\mu$ g/ml of hydrocortisone, 3  $\mu$ g/ml of insulin and 1  $\mu$ g/ml of epidermal growth factor. For attachment of the cells, a 1:1 mixture of MX83 medium and DME containing 10% FBS but no added growth factors was used. The hepatocyte cultures reached confluency ~48 h after plating. Each day, starting 24 h after plating, some of the coverslips were fixed with methanol at room temperature for 5 min, and stained with the appropriate antibodies as described below. Five days after plating, a "wound" (4 mm wide) was scratched into the confluent lawn of hepatocytes using a rubber policeman. The MX83 culture medium was changed 48 h later when a maximum number of cells near the edges of the wound were observed to be in S phase. BrdU was added to the cultures at a final concentration of 10  $\mu$ M. After 30, 60, and 180 min of exposure, respectively, treated coverslips were washed with PBS, fixed, and processed for immunofluorescence analysis. Three coverslips obtained from the wound experiments were used to test for albumin expression by the cultured hepatocytes using immunolabeling with anti-albumin antibodies (Miles Laboratories, Inc., Naperville, IL).

### Immunofluorescence Microscopy of BrdU and Gap Junction Protein on Separate Sections

Immunolabeling of incorporated BrdU or of the HJP28 in liver was carried out on 6–8- $\mu$ m-thick cryosections placed on round glass coverslips. The labeling procedure for the HJP28 followed the scheme described recently by Dermietzel et al. (9). In brief, sections were fixed in absolute ethanol for 15 min at –25°C. After rinsing in PBS the specimens were first incubated in 0.1% BSA in PBS for 15 min to reduce nonspecific labeling. They were then treated with either 35  $\mu$ l of affinity-purified rabbit anti-HJP28 (1  $\mu$ g/ml) (23) for immunolabeling of gap junctions or with commercial monoclonal anti-BrdU (Becton-Dickinson & Co., Paramus, NJ), diluted 1:50. In control slides the specific antibodies were replaced by rabbit or mouse IgG (5  $\mu$ g/ml). The secondary antiserum (1  $\mu$ g/ml) consisted either of affinity-purified, FITC-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc.) or TRITC-conjugated goat anti-mouse IgG (Miles Laboratories, Inc.).

### Double Immunofluorescence

To visualize both the expression of HJP28 and DNA synthesis, cryosections of regenerating liver or cultures of hepatocytes on coverslips were fixed in absolute ethanol for 0.5 h at –20°C. The samples were then treated with 0.2 M HCl for 0.5 h at room temperature in order to produce single-stranded DNA molecules. This was followed by exposure to 0.1 M borate buffer to neutralize the acid. These conditions were determined experimentally to cause sufficient DNA denaturation for reproducible staining with anti-BrdU reagents while causing minimal reduction of the antigenicity of the HJP28 (see however, discussion of null cells). The anti-BrdU and rabbit anti-HJP28 antibodies were applied simultaneously to the sections, which were then incubated for 1 h at room temperature, followed by thorough rinsing in PBS. FITC-conjugated goat anti-rabbit IgG served as a label for the

gap junction-antibody complex and TRITC-conjugated goat anti-mouse for detection of antibodies bound to BrdU. After incubation with the secondary antibodies the specimens were rinsed with PBS, and placed in glycerol containing 0.1% *P*-phenylenediamine to protect against quenching (25). The coverslips supporting the stained sections or the plated hepatocytes were then mounted on microscope slides for study by epifluorescence.

### Measurement of Fluorescence Intensity

**Photometry.** Photometric measurement of fluorescent dye binding was carried out only on liver sections. A measure of the amount of HJP28 present in regions of the liver lobule showing different levels of DNA synthesis was obtained by measuring separately green fluorescence (HJP28) and red fluorescence (BrdU) with a Leitz universal microscope equipped with a MPV28 photometer unit and appropriate filter combinations. The photometer was calibrated with a physical standard and the fluorescence measured at an exposure time of 1 s. The intensity of immunolabeling was determined by consecutively measuring the emissions of green and red fluorescence through an aperture (60  $\mu$ m diam) which was placed over randomly selected areas. These areas were rated for proliferative activity depending on whether they showed intense DNA labeling or contained no labeled nuclei. An area was considered to be in a state of "high proliferative activity" when a minimum of five nuclei within the field covered by the aperture exhibited BrdU labeling. Background fluorescence was measured in sections that had been treated with control IgG instead of specific antibodies. We found that the range of fluorescence varied from section to section. To allow comparisons, the data was calculated separately for each section. The statistical significance of the differences in the fluorescence of proliferative and non-proliferative areas were compared by the Chi<sup>2</sup>-test.

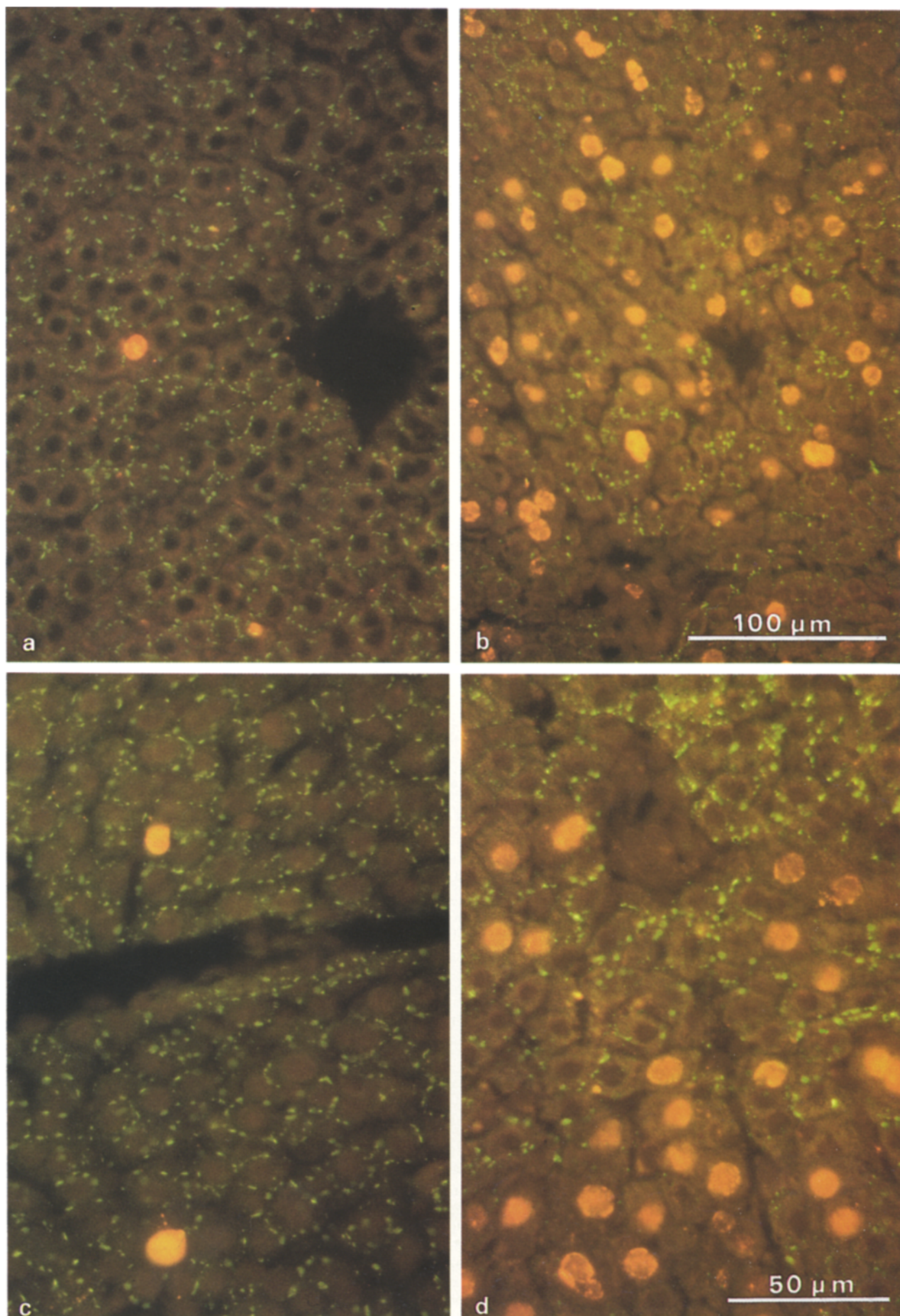
**Morphometry.** Fluorescence microscopy was carried out by means of a photomicroscope III (Zeiss) equipped with an epi-illuminator and appropriate excitation and barrier filters to detect both the green FITC-fluorescence and the red TRITC-fluorescence. An estimate of the amount of HJP28 could then be made by counting fluorescent spots representing the gap junction protein. Only clearly defined fluorescent spots were counted. The measurements were made on micrographs (400 $\times$ ) of double-immunolabeled specimens, so that BrdU labeling could also be assessed. Each sample was counted by two different observers. Cells were classified into three categories: (a) those with BrdU-labeled nuclei were said to belong to a proliferating population ( $P^+$  cells); (b) those without BrdU label but with HJP28 immunoreactivity were classified as nonproliferating ( $P^-$  cells); and (c) those cells that showed neither the BrdU labeling nor HJP28 immunoreactivity were classed as null cells (N cells). The presence and location of the N cells could be detected on phase contrast images of the same sections. Micrographs were taken on Kodak tri-X or Ektachrome 400 films.

## Results

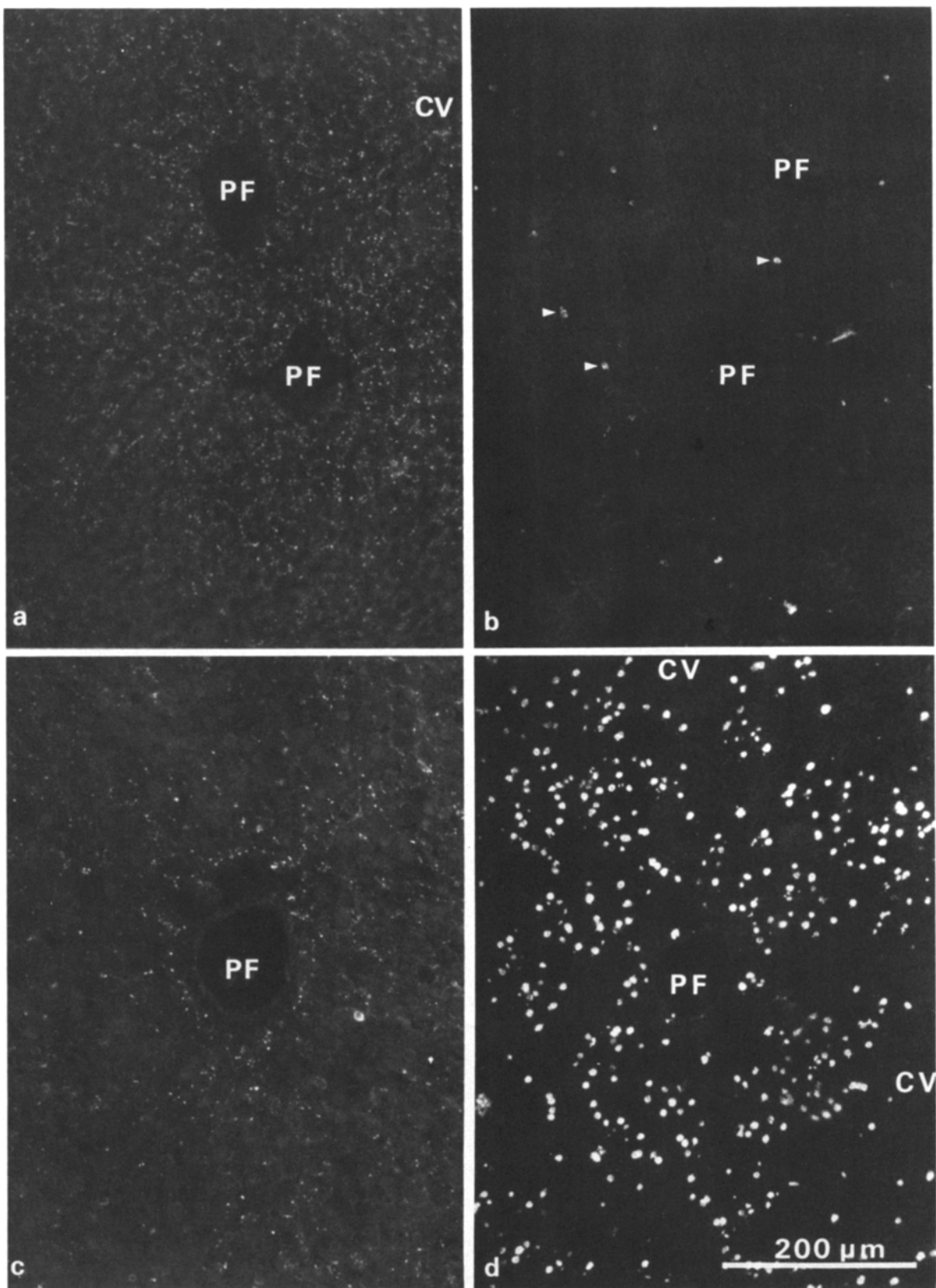
### Partial Hepatectomy

The mammalian liver consists of lobules, formed by radially arranged plates of hepatocytes, each separated from neighboring plates by specialized capillaries, the sinusoids. Hepatocytes in different positions in the lobule behave differently under physiological conditions, including response to mitotic stimuli (33). There are very few cells that show BrdU incorporation in control animals exposed to this nucleotide analog for 7 h (Figs. 1, *a* and *c*, and 2 *b*). Those rare cells that do take up BrdU are randomly located in the lobule. The gap junction protein HJP28, on the other hand, is found in essentially all cells of the lobules. The staining pattern

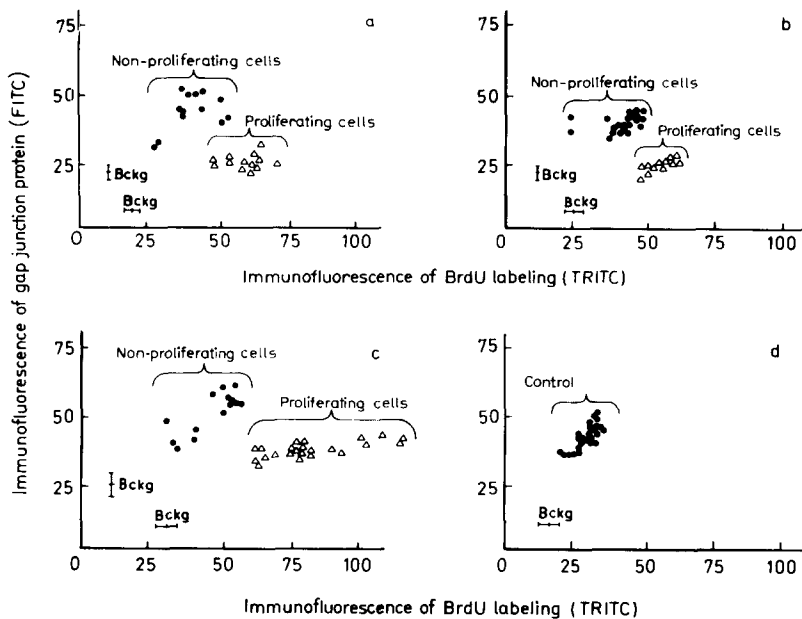
**Figure 1.** Double immunofluorescence showing both gap junctions stained with affinity-purified, anti-HJP28 antibodies, and cells that have synthesized DNA, detected by incorporation of BrdU which is then detected using a monoclonal antibody. Both *a* and *c* show the appearance of cryostat sections through liver from a sham-operated control. HJP28, seen by the green fluorescence of FITC bound to the second antibody has a punctate distribution, presumably because it is discretely localized in gap junctions, and is found only at the periphery of the cells. Those few cells in the control that have incorporated nucleotides in their DNA are seen by the red fluorescence of tetramethyl rhodamine. 26 h after partial hepatectomy (*b* and *d*) there are many more cells that have incorporated the nucleotide analog used as a marker for DNA synthesis. It is clear even from visual inspection that these  $P^+$  cells have suffered a significant loss of HJP28 immunoreactivity, while cells that have not gone through S phase, as judged by the lack of staining of their nuclei, have as many labeled junctions as the controls.







**Figure 2.** Indirect immunofluorescence of HJP28 and BrdU labeled independently on consecutive sections. (*a* and *b*) Sections from control animals showing the general distribution of HJP28 within a liver lobule (*a*), while documenting nuclear BrdU incorporation in a consecutive section (*b*). There is a low frequency of nuclear labeling (*arrowheads*). (*PF*) Portal fields. (*c* and *d*) Sections from partially hepatectomized animals 26 h after the operation. There is a drastic decrease in immunoreactive gap junction protein (*c*). Only a narrow girdle of cells around the portal field still shows a significant level of labeling. Within the lobule, BrdU labeling is the least intense near the periportal field (*PF*), and the central vein (*CV*) (*d*).



**Figure 3.** Plots of photometrically measured immunofluorescence of HJP28 immunoreactivity (FITC fluorescence), and BrdU labeling (TRITC fluorescence). (a–c) The intensity of light emission from three different sections obtained from livers after partial hepatectomy. (d) The data obtained from a control animal (sham operated). Open symbols ( $\Delta$ ) indicate the intensity of FITC and TRITC fluorescence from areas revealing a high proliferative activity, and closed symbols ( $\bullet$ ) label the fluorescence obtained from nonproliferating areas, respectively (for further details see Materials and Methods). The intensity of the light emission was determined by consecutively measuring the TRITC fluorescence and FITC fluorescence within the same areas covered by an aperture of 60  $\mu$ m in diameter. Background fluorescence (*Bckg*) was measured in sections from parallel incubations where the specific first antibodies were replaced by appropriate control IgG. The intensity of background fluorescence is indicated by the bar markers. Each bar marker is indicative of 20 measurements of background fluorescence. The grouping of the data was tested for significance using a  $2 \times 2$  contingency table.  $\chi^2$  proved significant at a  $P < 0.05$  level for all the sections. Ordinates and abscissae are in arbitrary fluorescence units.

delineating HJP28 on hepatocytes was, as expected, punctate and confined to the periphery of the cells (Figs. 1, *a* and *c*, and 2 *a*). In partially hepatectomized animals, increased numbers of BrdU labeled cells are already found at the earliest time period studied (19 h after partial hepatectomy). Because cells are exposed to BrdU for 7 h before samples are collected, these results show that hepatocytes could be entering S phase 12-h postoperatively or possibly even earlier. The cells labeled at 19 h are found preferentially in the periportal zone at the periphery of the lobules; however, an irregularly shaped rim of cells, immediately adjacent to the portal tract, remains unlabeled. Labeled cells are even more abundant 21 and 24 h after the operation. At these time points cells located more centrally in the lobule also have participated in DNA synthesis. Between 26 to 28 h after the operation more than two-thirds of the liver lobule contain cells that have synthesized DNA. Even at these times labeled cells are rarely in the most central zone or in the vicinity of the periportal field (Figs. 1, *b* and *d*, and 2 *d*). Labeled cells often appear grouped in clusters separated by fields of unlabeled hepatocytes (Fig. 1 *d*). A perceptible decrease in the number of labeled cells is seen at the 34-h time point, indicating that fewer cells entered mitosis between 28 and 34 h after hepatectomy than did earlier.

We found a reciprocal correlation between the number of cells entering mitosis, as shown by the BrdU incorporation, and the immunofluorescent labeling of HJP28 at all stages after partial hepatectomy. Because of the size of the hepatic lobule, the overall distribution of fluorescence is most readily appreciated at low magnifications but the low numerical aperture of the objectives needed makes it difficult to obtain satisfactory excitation for both labels simultaneously. We therefore labeled BrdU and HJP28 on consecutive sections for low magnification studies (Fig. 2, *a–d*). The conclusions

reached were confirmed by studies at higher magnification where both signals could be observed on the same section. The lobular distribution of the immunoreactivity of HJP28 is inversely related to that of the BrdU labeling. Cells adjacent to the periportal tract and those in the most central portions of the lobules consistently exhibited a low incorporation of BrdU and high intensity of HJP28 immunoreactivity (Figs. 1, *b* and *d*, and 2, *c* and *d*). In an attempt to quantitate this reciprocal relationship, we carried out a photometric analysis of randomly selected proliferating and nonproliferating areas (defined by the criteria indicated in Materials and Methods). Two distinct clusters of data points are found when plotting the results of these measurements on livers of partially hepatectomized rats. One of these represents cells with few junctions but a high incorporation of BrdU ( $P^+$ ). The other represents normal, quiescent cells ( $P^-$ ) which are also found in control liver (Fig. 3), where they represent the overwhelming majority.

By morphometric analysis of double-immunolabeled sections, we found that those cells that had incorporated BrdU ( $P^+$  cells), on the average, had the equivalent of  $0.52 \pm 0.1$  immunoreactive spots (junctions) of HJP28 per cell. This number probably does not truly reflect the presence of gap junctions in cells that are synthesizing DNA, but rather is due to the fact that some  $P^+$  cells are adjacent to  $P^-$  cells. In a small number of such cases (5%) one cannot be certain with which cell ( $P^+$  or  $P^-$ ) the fluorescent spots that denote the presence of HJP28 are associated. In contrast, cells without BrdU labeling ( $P^-$ ) displayed  $9.3 \pm 3.9$  spots (junctions) per cell. 26 h after partial hepatectomy, when the number of junctions is near its minimum as shown by electron microscopic and biochemical criteria, a little less than half of the cells can be designated as  $P^+$ , i.e., they had incorporated nucleotide analog into their DNA, and incidentally also

were found to have a greatly reduced junction complement. At the same time period only 1% or so of cells in the sham-operated control are P<sup>+</sup> (Table I).

### Tissue Culture Experiments

Cultured embryonic hepatocytes offer another opportunity to study, *in vitro* this time, a population of dividing cells. Generally speaking, it was found that when cell proliferation was high, expression of the gap junction protein was almost nondetectable. Well-defined regions of immunoreactive HJP28 could be found only after 24 h of culture. Expression of detectable gap junction protein occurs first in clusters of cells. Concurrently, there is significantly less BrdU labeling at these sites (Fig. 4, *a* and *b*). From these foci, expression of HJP28 progresses radially outwards increasing until a saturation is reached at ~48 h after the start of the culture. As labeling of gap junctions increases, the number of BrdU-labeled cells decreases (Fig. 4 *c*). These findings closely parallel the observations made *in vivo* (Fig. 1). The inverse relationship can also be seen in cultures of confluent hepatocytes caused to divide by mechanical disruption (wounding) (18). When a wound is produced in a lawn of the confluent cells 48 h after plating, there is an increase in the number of BrdU-labeled cells flanking the wound 24 h later. This is temporally coincident with a pronounced decrease in gap junction staining (Fig. 4 *d*). Counting of HJP28-positive sites (junctions) indicated  $1.07 \pm 0.85$  spots per cell in proliferating vs.  $7.33 \pm 1.43$  in nonproliferating areas, a five to seven-fold reduction. The three categories of hepatocytes (P<sup>+</sup>, P<sup>-</sup>, N) seen in regenerating liver can also be identified in a wound area. An increase in the number of P<sup>+</sup> cells and concomitant decrease in P<sup>-</sup> cells is already found after exposure to BrdU for 0.5–3 h before fixation (Fig. 5).

The arginine-free medium used in these experiments selects against growth of fibroblasts; however, to ascertain whether the cells lining the sides of the wound were hepatocytes or other cells (i.e., fibroblasts, endothelial cells), we labeled the specimens with anti-albumin. More than 90% of the total number of 540 BrdU-labeled cells screened reacted positively, and thus are believed to represent hepatocytes, although the immunoreactivity with anti-albumin was reduced as compared with that seen in normal cells.

### Discussion

Rapid changes of the number and size of gap junctions have been observed in the liver lobule remaining after partial hepatectomy (45, 46). It has proved difficult, however, to correlate the presence of gap junctions between hepatocytes with the cell cycle in regenerating liver. Part of the problem is the fact that the liver, although it consists largely of one cell type, is functionally not homogeneous. It is well established that tritiated thymidine (<sup>3</sup>HT) incorporation does not

**Table I. Percentage of Cells in Different States in Partially Hepatectomized Animals (26 h after Operation) and Control Animals**

Type of cell	Operated		Control	
	No.	%	No.	%
P <sup>+</sup>	508	44.2 ± 5.4	18	1.2 ± 0.9
P <sup>-</sup>	559	48.6 ± 4.8	1485	96.1 ± 2.4
N	82	7.1 ± 3.7	65	2.3 ± 1.5

The fractional distribution of the three classes of cells seen in double immunolabeling experiments are displayed. Data from partially hepatectomized animals and sham-operated control animals are tabulated. Cells showing BrdU incorporation are indicated as P<sup>+</sup> cells. P<sup>-</sup> cells are hepatocytes showing gap junction protein immunoreactivity without nuclear labeling. N cells show no detectable BrdU labeling or gap junction protein immunoreactivity. The percentages recorded represent the mean ± SD of measurements made in four separate experiments.

occur throughout a lobule at any one time but rather spreads, wavelike, from the periphery (periportal region) toward the middle (central vein) (34). In morphological and physiological studies of the gap junction however, it is difficult to know with any precision the location of the sample within the lobule (46) or the position of individual cells in the cell cycle. The double-labeling approach used here has enabled us to partially overcome such problems by allowing us to quantify the presence of gap junctions in hepatocytes whose position, with respect to the lobule and the S phase of the cell cycle, is known with reasonable accuracy.

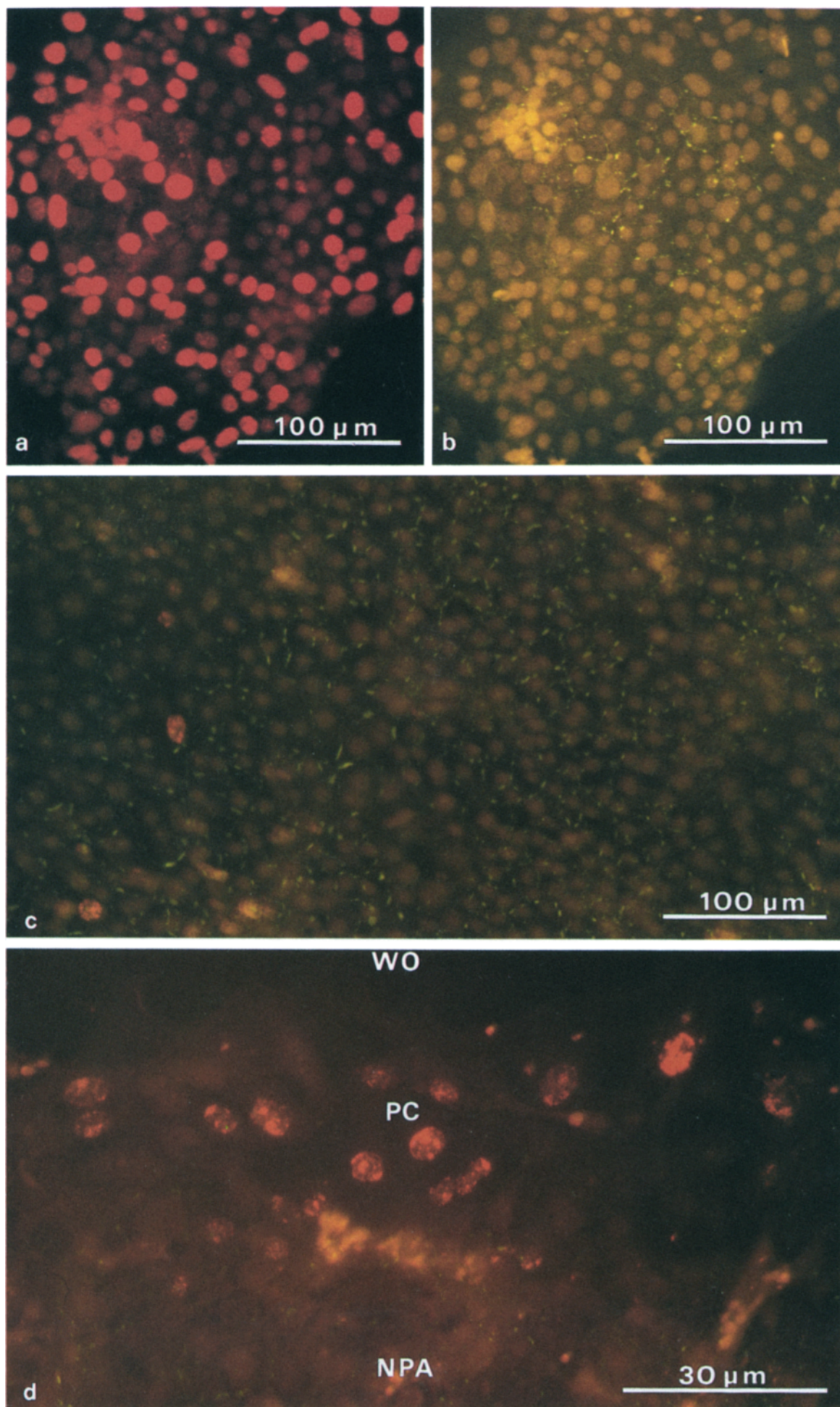
### Validity of the Labeling Technique

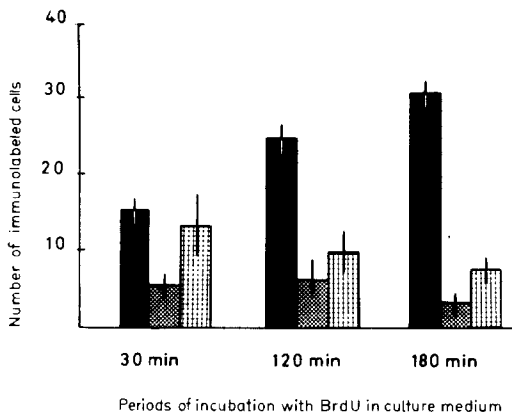
**BrdU Labeling.** There are potential problems inherent in the use of BrdU as an indicator for cells that synthesize DNA. Some of these problems are common to all incorporation techniques, others specific to the use of BrdU. Thus, the fact that not all hepatocytes incorporate BrdU at any one time is not likely to be an artifact due to the use of this nucleotide analog, since nonuniform uptake is also seen after tritiated thymidine labeling (37). As detailed in the section on Materials and Methods, we chose to use a 7-h exposure time to the analog to insure sufficient uptake by cells. At any one time point the experimenter is therefore looking at that fraction of cells that has entered the S phase at some (undetermined) point during this 7-h time span. Some cells that contain only small amounts of detectable label could either have left the S phase after exposure for only a few hours and now be approaching M, or alternately, may have only recently entered the S phase. As a result there is some degree of uncertainty about the temporal coupling of junction loss and position in the cell cycle.

A problem specific to the use of BrdU is the possibility that it may interfere with cell division and/or gene expression (26). Previous authors, however, have shown that it can be used for cell cycle studies (11). In addition, we find that the

**Figure 4.** Double immunolabeling of liver cell cultures. Labeling of BrdU incorporated in DNA and HJP28 was as in Fig. 1. (*a* and *b*) A colony of hepatocytes 36 h after plating. At this phase, proliferative activity is high (*a*) and gap junction protein expression occurs only in the center of the colonies (*b*) where only a small number of BrdU-positive nuclei are discernible. (*c*) Confluent layer of cultured hepatocytes 48 h after plating. Note that the pattern of HJP28 and BrdU immunoreactivity is almost identical to that of the pattern found in control animals. (*d*) Wounding experiment showing the wound (WO), the proliferative marginal compartment (PC), and part of the nonproliferative area (NPA). Note that the area containing proliferating cells is almost devoid of HJP28 immunoreactivity, while the rest (NPA) reveals well-defined immunolabeling of this gap junction protein.







**Figure 5.** Histogram depicting the number of immunolabeled cells adjacent to the wound in a lawn of confluent hepatocytes classified according to the criteria described under Results. Cells were counted in the proliferative marginal compartment flanking the wound (see Fig. 1 c). Three different incubations with BrdU were performed (periods of exposure indicated on the x-axis), and data were pooled from three experiments of each labeling interval. The diagram shows a significant increase of BrdU-labeled cells P<sup>+</sup> cells at longer labeling periods while the number of P<sup>-</sup> cells decreases. (■) P<sup>+</sup> cells; (▨) N cells; (▤) P<sup>-</sup> cells.

replication kinetics of the liver in partially hepatectomized animals are similar after BrdU to those after labeling with tritiated thymidine (34). Neither does there seem to be interference with HJP28 synthesis or expression under our experimental conditions, since the pattern of the HJP28 seen in partially hepatectomized animals that were not being subjected to BrdU is identical to that seen after its administration. The reduction in the number and relative size of gap junctions and the amount of HJP28 has also been found by freeze-cleaving (45, 46) or cell fractionation techniques (12, 41).

**Labeling of HJP28.** If one is to attempt to define a functional correlation between the presence of gap junction protein and the control of cell division it is important that the antibodies used be capable of detecting physiologically “competent” molecules. It is possible to imagine that the HJP28 could be in the form of an inactive precursor sharing an epitope with the physiologically active protein, in which case the disappearance of junction protein early in the events leading to regeneration would be less interesting to interpret. In the experiments described, however, we know that the same antibody preparation also blocks communication (28; Traub, O., J. Look, R. Dermietzel, and K. Willecke, manuscript in preparation), i.e., that at least a fraction of the immunofluorescence signal is due to the presence of physiologically active junction protein. Immunostaining using gold-labeled second antibody on sucrose-embedded frozen sections prepared for the electron microscope has shown that the antibody binds to all morphologically recognizable junctional domains, but not to nonjunctional plasma membranes (9, 10). We therefore believe that, as a first approximation, the punctate staining observed represents physiologically functional gap junctions. It is not possible at present to eliminate the possibility that some of these junctions could be partially or wholly in a nonconductive state.

Other proteins have been identified as part of gap junctions and one could therefore argue that they, rather than HJP28,

represent the channel-forming protein. If this were the case one could view the change in HJP28 as incidental. There is evidence for the presence in mouse liver gap junctions, and to a much smaller extent rat liver junctions, of at least one additional protein, of *M<sub>r</sub>* 21 kD. Using affinity-purified antibodies Traub and co-workers (Traub, O., J. Look, R. Dermietzel, and K. Willecke, manuscript in preparation) have now shown that both proteins decrease after hepatectomy, and both seem to colocalize to the same junctional plaques (31a). Taken together with previously published electron microscopic observations showing a decrease in the number of junctional plaques, this would suggest that only one type of cell junction is responsible for cell-cell coupling. The protein of *M<sub>r</sub>* 16 kD described by Pitts and collaborators (11a) also decreases after hepatectomy, but it is not yet clear if the time course is identical to that described here.

### Comparison of Different Techniques to Measure the Junction Complement

In the present set of experiments, about half of the cells in regenerating liver are found to be in a proliferative state (P<sup>+</sup>), i.e., not to express HJP28 but to have incorporated BrdU. This must be compared with the data obtained by morphometry of freeze-cleave material (29). Here ~5% of the hepatocytes are found to totally lack communication with their neighbors at ~28–30 h after hepatectomy (data from Table IV in reference 29) and thus can be assumed to be junctionless. At least these many cells would be seen as P<sup>-</sup> cells. It is likely, however, that electrophysiological detection of coupling is much more sensitive than enumerating junctions, either by freeze-cleaving or immunofluorescence. If the present and the 1980 sets of data are directly comparable (there may be differences due to a number of uncontrollable factors with experiments done so many years apart under different conditions), one would conclude that electrophysiology would be about 10 times as sensitive as the morphological approaches, not an unreasonable number.

Another way to approach the problem is to compare the number of junctions per cell in the two sets of data. Yancey et al. (46) and Meyer et al. (29) found that there are 0.08 junctions/μm<sup>2</sup> in control liver as against 0.008/μm<sup>2</sup> in regenerating liver 28 h after hepatectomy. If this is combined with the area of hepatocyte in contact with its neighbors as calculated by us (29), one concludes that there may be 48 junctions/cell in normal and five in regenerating livers. Again this compares satisfactorily with the data presented here (10 junctions/cell in controls and 0.5/cell in livers of experimental animals), if one takes into account the fact that the FITC-labeled junctions, enumerated in the present study, would be those junctions near the optical slice determined by the numerical aperture of the objectives used. Junctions at the “top” or “bottom” of a prismatic cell would be out of focus and not be counted. The present data also fit with the estimate of 13–14 “communicating interfaces” (i.e., hepatocyte faces in contact with a neighbor and containing at least one junction) (29), which were found between cells in control livers, vs. 0.5–1.5 communicating interfaces in the regenerating organ.

### The N Cell Population

The observed differences in the BrdU uptake and stainability by fluorescent antibody against HJP28 leads us to distinguish



among three classes of cells: (a) proliferating cells ( $P^+$  cells), which incorporate BrdU; (b) nonproliferating cells ( $P^-$  cells), which do not incorporate BrdU but contain HJP28; and (c) null cells (N cells), which do not bind either label.

The existence of N cells was surprising and a potentially important factor in interpreting the data. We find, however, that at least a fraction of N cells is produced by initial treatment of the sections with HCl in order to produce the single-stranded DNA necessary for the immunolabeling of incorporated BrdU. This treatment causes a 20% reduction in immunofluorescence of the HJP28 in tissue sections or in slides of cultured cells when compared with untreated specimens (data not shown). We also found that when sections from control liver are treated with the same concentration of HCl, there is an increase of N-cells, comparable to that observed in specimens of regenerating liver. Altogether these observations decrease the likelihood that N cells will prove physiologically significant.

### Cell Cycle-related Disappearance of Gap Junctions

As discussed above, previous data on regenerating liver have shown in a general way the relationship between loss of junctions and cell division. The present data allow one to be more precise about the relationship. They suggest: (a) it is those cells that have been activated to enter the division cycle that lose their junctions; (b) the loss of junctions involves, in part at least, the loss of junctional protein, although changes in configuration that could lead to changes in immunoreactivity could give the same result; (c) the decreasing number is an early event, and thus could be part of a chain of causal events; (d) the junction depression persists for most if not all of the division cycle. The conclusions to be drawn from the study of the regenerating liver are supported by the data gathered by the wounding of confluent hepatocyte cultures. Here there is a decrease in the expression of HJP28 only at the very edges of the wound, in those cells that are stimulated to proliferate. The surrounding cells retain their junction protein and remain in  $G_0$  or  $G_1$  phase. The correlation between junction loss and cell division is maintained during the 180-min period of BrdU application, suggesting a strong linkage between the two.

While these data could be used to support a causal link, data on other proliferating systems do not permit an easy generalization to be made, with some in general agreement with ours and others not as easily reconciled. Thus Spagnoli et al. (40), working on the regenerating arterial endothelium, have reported a loss of gap junctions in the zone of high proliferative activity as determined by autoradiography. Very recently Goodall and Maro (15) convincingly demonstrated that a loss of junctional coupling occurs during mitosis of early mouse embryos. On the other hand, Bjerknes et al. (5) demonstrated a high degree of dye coupling between the basal epithelial cells of the crypts in the small intestine of the mouse. These cells are known to exhibit a high degree of proliferative activity. Whether cells in the generative pool or cells already becoming differentiated were impaled is not clear, however. A more clearcut instance is that of the trachea. When tracheal epithelial cells are stimulated to divide, gap junctions begin to appear at the end of  $G_1$  phase, and reach their highest numbers in the S phase. In this case a dis-

appearance of gap junctions in the M phase was reported (16).

We can only speculate at present about the functional meaning of the reduction in gap junctions correlated with DNA synthesis in regenerating hepatocytes. Since gap junctions are channels that permit widespread exchanges of low molecular mass substances between cells, a loss or a qualitative shift of junctional coupling could result in functional segregation of the cell from its neighbors. There are major changes in the synthetic activity of hepatocytes in their response to a proliferative stimulus. Most of these regulative processes of hepatocytic growth are assumed to be based on transcriptional mechanisms (for review see Nakamura and Ishihara [31]) and a considerable number of cytoplasmic factors and molecules show reciprocal regulation during the shift from growth to the resting state (30). The temporary withdrawal from the physiological syncytium of normal liver, however, could be an effective way to allow cell division without interfering with the homeostatic balance within the nonproliferative cell population. Alternatively of course, positive signals could be interrupted and thus lead to entry into the division cycle.

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